Binding Sites of Cholinesterases

ALKYLATION BY AN AZIRIDINIUM DERIVATIVE

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1. Alkylation of acetylcholinesterase and butyrylcholinesterase by 2-chloro-*N*-(chloroethyl)-*N*-methyl-2-phenylethylamine was observed. This alkylating agent was more potent than related compounds previously described, and less stable (half-life 8.5 min. at 23°). 2. Alkylation had effects on hydrolysis of substrates varying from activation for indophenyl acetate to inhibition for acetylcholine, and intermediate effects with five other substrates. The effects were on $V_{\max}$, and not K_m . 3. Alkylation caused a variety of changes in sensitivity to inhibition by five carbamates, five organophosphates and four other inhibitors, varying from total protection against tetraethylammonium to mildly enhanced sensitivity to urea. 4. The findings suggested the existence of three binding sites, one of which was anionic and another hydrophobic, in addition to the esteratic site.

The picture of the active site of AChE* that is most widely accepted is that proposed by Wilson and collaborators (Wilson, 1960), i.e. that there is an 'esteratic site' that becomes acetylated during the reaction with acetylcholine, and also a nearby 'anionic site' that coulombically binds the acetylcholine before the acetylation reaction. Organophosphates and carbamates react in an analogous way, undergoing a binding step followed by a phosphorylation or carbamoylation step. The question that this paper considers is: does the binding step of all substrates and inhibitors involve a common binding site, or are there numerous available binding sites?

Recent work has stressed the importance of hydrophobic interactions in binding organophosphates to acetylcholinesterase (Brestkin *et al.* 1964; Bracha & O'Brien, 1968*a,b*) and has pointed to the existence of at least one, rather clearly defined hydrophobic area near the esteratic site. The existence of separate hydrophobic and coulombic binding zones could explain the surprisingly small difference in reactivity of charged and uncharged substrates [such as acetylcholine and its analogue in which the nitrogen atom is replaced by a carbon atom (Adams & Whittaker, 1950)] and inhibitors [such as Amiton, $(C_2H_5O)_2PO \cdot S \cdot CH_2 \cdot CH_2 \cdot N \cdot (C_2H_5)_2$ and its carbon analogue (Bracha & O'Brien, 1968*a*)]. However, it would introduce difficulties in attempts to evaluate coulombic contributions by

comparing such charged and uncharged compounds, either by using pairs of such compounds or by examining the pH-dependence of reactivity of ionizable compounds (Wilson & Bergmann, 1950).

Belleau & Tani (1966) described a new type of anti-cholinesterase, and suggested that it irreversibly alkylated the enzyme after attachment to the anionic site. This compound was the *NN*-dimethyl-2-phenylaziridinium ion, prepared by adding the corresponding 2-chloroethylamine to water. Further, Purdie & McIvor (1966) reported that the enzyme alkylated with *NN*-dimethyl-2-phenylaziridinium ion, although partially inhibited as far as hydrolysis of acetylcholine was concerned, was 100% activated as far as hydrolysis of indophenyl acetate was concerned. Also the susceptibility to inhibition by Amiton, was decreased or abolished, whereas Sarin, $(isoC_3H_7O)(CH_3)PO \cdot F$, Soman, $[(CH_3)_3C \cdot CH(CH_3)O](CH_3)PO \cdot F$, and TEPP, $[(C_2H_5O)_2PO]_2O$, were still potent inhibitors. It was suggested that the alkylating agent reacted with the anionic site, and that only Amiton (of the four organophosphates above) bound obligatorily to that site, as did acetylcholine. By contrast, indophenyl acetate, Sarin, Soman and TEPP presumably could react with the esteratic site without requiring to be bound to the anionic site.

The above report appeared to provide a remarkably unambiguous classification of compounds, and implied that two separate binding zones existed on the enzyme, with relatively little overlap. Studies were therefore undertaken of the effect of treatment

* Abbreviations: AChE, acetylcholinesterase; MCP, 2-chloro-*N*-(chloroethyl)-*N*-methyl-2-phenylethylamine.

Scheme 1. Ph, Phenyl; R₁, -CH₃; R₂, -CH₂·CH₂Cl.

Fig. 1. Rate of alkylation of AChE (●) and butyrylcholinesterase (○) by 0.01 mM-MCP at 25° at pH 8. Substrate was added after incubation.

Fig. 2. Concentration-dependence of alkylation of AChE by MCP at 25° at pH 8 during 2-25 hr. incubation. Substrate was added after incubation.

in iced equimolar sodium hydroxide solution (to neutralize its hydrochloride salt), then added to the titration vessel at 25°. Production of acid decreased exponentially; the half-life was 7.6 min. at pH 8 and 8.2 min. at pH 9. The findings are readily interpretable in terms of the scheme of Chapman & Triggle (1963), shown in Scheme 1. Presumably the aziridinium ion is the active alkylating agent.

AChE was alkylated by incubation for 2-25 hr. with 1.0 mM- or 0.1 mM-MCP at room temperature (about 23°). After this time, all the excess of MCP had disappeared, the activity of the enzyme toward acetylcholine was decreased (in fact almost eliminated with 1.0 mM-MCP) and that toward indophenyl acetate was increased, as shown in Table 1.

Purdie & McIvor (1966) found slowly progressive effects of *NN*-dimethyl-2-phenylaziridinium on AChE, extending over more than 5 hr., after which time the hydrolysis of indophenyl acetate was

Fig. 3. Effect of the presence of substrate (acetylcholine) during incubation of AChE and butyrylcholinesterase with MCP at 25° at pH 8. Inhibitions reported were those 10 min. after the enzyme was mixed with substrate and inhibitor. (a) Activity without MCP: ○, AChE; ●, Butyrylcholinesterase. Units of activity were arbitrary (b) Inhibition by MCP: ○, AChE by 0.1 mM-MCP; △, AChE by 0.01 mM-MCP; ●, butyrylcholinesterase by 0.01 mM-MCP.

unaffected, and that of phenyl acetate was inhibited by 50%, that of indoxyl acetate by 55%, and that of acetylcholine and acetylthiocholine by 65%. I found with MCP a much more rapid and complete response; indeed, the brief half-life of the MCP excluded the possibility of a steadily progressive reaction. Fig. 1 shows that at 0.01 mM-MCP, the alkylation (measured by blockage of acetylcholine hydrolysis) proceeded much faster for AChE than for butyrylcholinesterase. The concentration-dependence of alkylation was far less than for more stable compounds (Fig. 2). Alkylation was slowed by the presence of substrate (Fig. 3), the protection being less pronounced with butyrylcholinesterase than with AChE.

Other substrates were studied (Table 1); alkylation at either of two concentrations of MCP produced an enzyme activated for hydrolysis of indophenyl acetate, and inhibited modestly for that of *p*-nitrophenyl acetate and much more strongly for that of other substrates, acetylcholine being the most affected.

For three substrates (whose hydrolyses were the most activated, the most inhibited, and a middle type) the question of whether the above effects were due to changes in K_m or V_{max} was studied. Table 2 shows that in every case K_m was little changed, and virtually all the effects were on V_{max} . It was necessary to compare the absolute V_{max} values for

these compounds (see the Discussion section). The relative proportions of the $V_{\max}$ for 1 unit of enzyme with acetylcholine:indophenyl acetate:*p*-nitrophenyl acetate were 225:1:4.5.

Table 1. *Effect of alkylation of AChE by MCP on hydrolysis of various substrates*

All substrates were at 1.0 mM final concentration, except *p*-nitrophenyl acetate (0.33 mM).

Substrate	Activity (% of control value)	
	Alkylated by 1.0 mM-MCP	Alkylated by 0.1 mM-MCP
Indophenyl acetate	161	129
<i>p</i> -Nitrophenyl acetate	78	72
2-Naphthyl acetate	14	43
Indoxyl acetate	11	29
Phenyl acetate	7	18
1-Naphthyl acetate	5	16
Acetylcholine	0.7	9

Organophosphates gave progressive inhibition of the enzyme, from which a bimolecular rate constant k_i could be calculated as described by Aldridge (1950). Carbamates caused inhibition that increased until a plateau of inhibition was reached, and this plateau value is reported here. The other inhibitors, being reversible, gave prompt and unchanging inhibition.

Tables 3 and 4 show that the potency of all inhibitors was much decreased by alkylation of the enzyme. Of the carbamates and phosphates by far the most affected was Amiton, whose reactivity declined 913-fold even with mildly alkylated enzyme. The inhibitory potency of choline and tetraethylammonium was abolished by alkylation of the enzyme.

The final question was: how does the choice of substrate affect the estimate of reactivity with organophosphates (the phosphorylation reaction occurring, as in all cases in the present study, in the presence of substrate)? The question was studied with the organophosphate most affected by alkyl-

Table 2. *Effect of alkylation by 0.1 mM-MCP on K_m and $V_{\max}$ for AChE*

The $V_{\max}$ rate units are arbitrary.

Substrate	Control AChE	Alkylated AChE	Alkylated/control ratio
Indophenyl acetate	$V_{\max}$ 27.4	41.7	1.5
	K_m $7.15 \times 10^{-4} M$	$7.15 \times 10^{-4} M$	1.0
<i>p</i> -Nitrophenyl acetate	$V_{\max}$ 98.0	50.8	0.52
	K_m $1.22 \times 10^{-3} M$	$1.12 \times 10^{-3} M$	0.92
Acetylcholine	$V_{\max}$ 145.4	9.9	0.068
	K_m $2.13 \times 10^{-4} M$	$3.36 \times 10^{-4} M$	1.6

Table 3. *Effect of alkylation on susceptibility of AChE to inhibition by steady-state and reversible inhibitors*

All the results were obtained with indophenyl acetate as substrate at 25° and pH 8. Alkylations, in the absence of substrate or inhibitor, were carried out for 2.25 hr. All except quaternary inhibitors were with ethanol (final concn. 0.12 M) and the reported inhibitions were by comparison with ethanol alone. Five replicates were commonly used, or less if the range was small.

	Inhibition (% of control value)		
	Control enzyme	Enzyme alkylated by 0.1 mM-MCP	Enzyme alkylated by 1.0 mM-MCP
Carbamates (0.014 mM)			
<i>O</i> -Isopropyl <i>N</i> -methyl <i>N</i> -phenylcarbamate (IPC)	85	26	5
1-Naphthyl <i>N</i> -methylcarbamate (carbaryl)	79	14	26
<i>m</i> -Trimethylammonium phenyl <i>NN</i> -dimethylcarbamate (neostigmine)	88	18	5
Eserine	97	26	35
3,5-Di-isopropylphenyl <i>N</i> -methylcarbamate (DIP)	96	38	65
Quaternary reversible inhibitors (75 mM)			
Choline	67	0	2
Acetylcholine	77	18	10
Tetraethylammonium	77	0	0
Others (1 M)			
Urea	12	32	

Table 4. *Effect of alkylation on susceptibility of AChE to inhibition by organophosphates*

	10 ⁻³ × Bimolecular rate constant, <i>k_i</i> (min. ⁻¹ mole ⁻¹ l.)		
	Control enzyme	Enzyme alkylated at 0.1 mM-MCP	Control/alkylated ratio
Paraoxon (diethyl <i>p</i> -nitrophenyl phosphate)	734	78.0	9
Guthoxon (<i>S</i> -3,4-dihydro-4-oxo-1,2,3-benzotriazin-3-ylmethyl <i>OO</i> -dimethyl phosphorothiolate)	481	38.8	12
Malaaxon [<i>S</i> -1,2-bis(ethoxycarboxyl)ethyl <i>OO</i> -dimethyl phosphorothiolate]	138	5.0	27
Amiton [<i>S</i> -(diethylaminoethyl) <i>OO</i> -diethyl phosphorothiolate]	114	0.125	913
PB 72 (<i>S</i> -5,5-dimethylhexyl <i>OO</i> -diethyl phosphorothiolate)	228	6.9	33

ation of the enzyme (Amiton) and that least affected (paraoxon), and by using control enzyme and also that partially alkylated with 0.1 mM-MCP. In the latter case it was necessary to use ten times as much alkylated enzyme as of control enzyme when acetylcholine was substrate, because of the low reactivity of alkylated enzyme towards acetylcholine.

With control enzyme, similar values for reactivity with paraoxon and Amiton were found with both substrates. Thus in duplicate runs with one enzyme preparation, *k_i* values (defined below and expressed as min.⁻¹l. mole⁻¹) for paraoxon were: 7.4 × 10⁵ and 10.9 × 10⁵ with acetylcholine; 7.9 × 10⁵ and 6.8 × 10⁵ with indophenyl acetate. Similarly for Amiton, *k_i* was 3.3 × 10⁵ and 5.0 × 10⁵ with acetylcholine; 1.3 × 10⁵ and 1.0 × 10⁵ with indophenyl acetate. But when alkylated enzyme was used the substrates gave very different values. Thus for paraoxon the *k_i* with acetylcholine as substrate was 10.1 × 10⁵ and 7.5 × 10⁵ (i.e. the same as with control enzyme), but with indophenyl acetate the values were 0.8 × 10⁵ and 0.75 × 10⁵ (i.e. about one-tenth of that for the control enzyme). With Amiton the effect was even more pronounced: for acetylcholine *k_i* was 1.9 × 10⁵, 2.0 × 10⁵ and 2.3 × 10⁵ (i.e. close to values for control enzyme); whereas with indophenyl acetate the values were 1.3 × 10² and 1.4 × 10² (i.e. about one-thousandth of the control values).

The above values show that the estimate of inhibitory potency of phosphates is not affected by choice of substrate in the normal enzyme, but is severely affected in the alkylated enzyme. They also show that a change caused by alkylation in susceptibility to phosphorylation is revealed when indophenyl acetate is substrate, but not when acetylcholine is substrate.

DISCUSSION

The phrase 'strongly alkylated' will be used below to describe the enzyme alkylated by 1 mM-MCP, and

'mildly alkylated' for enzyme alkylated by 0.1 mM-MCP.

For all substrates and for the anti-cholinesterases, the results can be discussed in terms of eqn. (1), which is equally valid for substrates, carbamates and phosphates:

where A is the acetyl, dialkoxyposphoryl or *N*-methylcarbamoyl group, and B is the leaving group. The first step is complex-formation, governed by $K_a = k_{-1}/k_{+1}$, the second step is acylation, phosphorylation or carbamoylation, and the third step is hydrolytic regeneration of the enzyme. Under the conditions employed $k_i = k_2/K_a$ (Main, 1964). Gutfreund & Sturtevant (1956) have shown for the precisely analogous case of chymotrypsin that:

$$K_m = \frac{k_{-1} + k_2}{k_{+1}} \cdot \frac{k_3}{k_2 + k_3} \quad (2)$$

Effects on substrates and on reversible inhibitors. Strong alkylation of the enzyme virtually abolishes its reactivity towards acetylcholine, or its ability to be inhibited by choline or tetraethylammonium. These three compounds have, like MCP, a quaternary ammonium group; it is suggested that all these compounds combine with a common site, which we may call the α-site, and which could be the same as the 'anionic site'.

It follows that the strongly alkylated enzyme, which has lost 99.7% of its ability to hydrolyse acetylcholine, has 99.7% of its α-sites blocked. We must now explain why it can hydrolyse six other substrates at 5% to 161% of control rates (Table 1); it appears that these various responses are reflections of variations in V_{max} , but not in K_m (Table 2).

The possibility that K_a could be affected by alkylation (i.e. that the affinities, $1/K_a$, of the various substrates were differentially effected) can be ruled out on three grounds. (a) It is extremely

unlikely that $K_a (=k_{-1}/k_{+1})$ could be changed without changing K_m ; the only way this could occur would be by a fortuitous counterbalancing shift in the residual terms of eqn. (2). (b) Alkylation affects $V_{\max.}$, which is computed for saturating conditions when the K_a step is unimportant. (c) One cannot account for the increased rate of hydrolysis of indophenyl acetate by a decrease of affinity.

Now $V_{\max.}$ is a reflection of k_2 or k_3 , whichever is rate-limiting. But the k_3 step is identical for all six substrates, for they all yield an acetylated enzyme. Consequently the greatly varying responses in $V_{\max.}$ must be due to varying effects upon k_2 , the acetylation step.

The fact that there are two classes of substrates, some whose k_2 is decreased and one whose k_2 is increased by occupation of the α -site by MCP, is explicable only if one assumes that they bind to two classes of site, which we may call β and γ respectively; these sites must of course be distinct from the α -site, which is already occupied. A simple hypothesis to explain why β -agents (i.e. those binding to the β -site) and γ -agents (those binding to the γ -site) are differentially affected is that alkylation causes a configurational shift that shifts the catalytic group (usually assumed to be a serine hydroxyl group) away from the β -site and towards the γ -site. Such a shift would of course affect k_2 and not K_a or k_3 , as the experimental findings require.

Effects on carbamate and phosphate inhibitors. No γ -agents have been found among the phosphates or carbamates. But clearly Amiton (Table 3) is an α -agent, as judged by its 913-fold decrease in rate constant even on mild alkylation of the enzyme; by contrast, the other phosphates suffer a modest decrease in rate constant and are classified as β -agents.

Among the carbamates, eserine, carbaryl and 3,5-di-isopropylphenyl methylcarbamate are clearly β -agents because their reactivity is mildly decreased by alkylation. The other two carbamates lose 95% of their inhibitory activity on strong alkylation of the enzyme, and so may well be α -agents; and indeed one of the carbamates, neostigmine, has a quaternary ammonium group like the other α -agents described above.

It is an oddity of those carbamates that are β -agents, that mild alkylation of the enzyme decreases the inhibition by them more than does strong alkylation. The mechanism behind this phenomenon is not clear, but the finding reinforces the distinction between α - and β -type carbamates.

Some alternative hypotheses. The total blockade, by alkylation of the enzyme, of α -site agents could be interpreted in a way other than that given above. Rather than blocking the α -site, alkylation might shift the α -site relative to the esteratic site so extensively that agents bound to the α -site could

not reach the esteratic site with their acylating groups. Such a hypothesis has the virtue of explaining all the results by postulating only one kind of effect. However, the marked protective effect of acetylcholine against alkylation (Fig. 3) is hard to explain by this hypothesis.

There are also alternative ways to explain the activation of hydrolysis of indophenyl acetate by alkylation of the enzyme. (a) Alkylation might reveal additional sites on the enzyme, but it would be remarkable if these should have the same K_m as the original sites. (b) Alkylation might prevent formation of hypothetical 'adverse binding'. If so, such adverse binding would have to occur with an affinity very similar to that for the 'successful binding', or else departures from Michaelis-Menten kinetics would be seen, as in the case for acetylcholine where 'adverse binding', involving formation of inactive ES_2 , is seen only at high substrate concentrations. In addition, the 'adverse' effect would have to show its effect on $V_{\max.}$ but not on K_m , i.e. the 'adverse' molecule would have to affect k_2 but not the affinity of indophenyl acetate for its 'successful-binding' site. (c) The hydrolyses of acetylcholine and indophenyl acetate might be catalysed by entirely different sites, and only the acetylcholine-hydrolysing site might be alkylatable. This alternative is, however, virtually excluded by the finding that in normal AChE, the inhibitory potency (k_1) of Amiton or of paraoxon is unaffected by the choice of substrate (acetylcholine or indophenyl acetate) employed for its assay, although the k_i of Amiton appeared to be a little greater with acetylcholine than with indophenyl acetate.

Finally, the possible existence of isoenzymes must be considered. Could MCP strongly inhibit a hypothetical acetylcholine-hydrolysing isoenzyme, mildly inhibit a *p*-nitrophenyl acetate-hydrolysing isoenzyme, and activate a third, indophenyl acetate-hydrolysing, isoenzyme? The possibility that three such isoenzymes exist in the unalkylated enzyme is made very improbable by the fact (stated above) that the rate constants for phosphorylation by paraoxon or Amiton were very similar whether assayed with acetylcholine or with indophenyl acetate.

It is concluded that these alternative hypotheses are relatively implausible.

Relation to reported effects of alkylammonium ions. A series of papers has described activation effects of tetraethylammonium ions on the reaction of AChE with substrates and inhibitors. Thus tetraethylammonium ions in the 1 mM range activated the hydrolysis of acetyl fluoride threefold (Metzger & Wilson, 1967), the hydrolysis of dimethylcarbamoyl fluoride 14-fold (Metzger & Wilson, 1963) and the inhibition of AChE by methylsulphonyl fluoride 30-fold (Kitz & Wilson, 1963). In terms of the

hypothesis of the present paper, the implication is that these are all γ -site reagents, and that binding of tetraethylammonium ions induces a configurational shift precisely as does alkylation by MCP. The suggestion by Metzger & Wilson (1967) is that 'the binding of the quaternary ammonium ion induces a change at the esteratic site which causes this site to react more rapidly with the acid fluoride'. This explanation is in accord with that proposed above, but is less specific. These findings are complicated by the observations that other alkylammonium ions have quite different effects: for instance, inhibition by methylsulphonyl fluoride is unaffected by tetrapropylammonium ion and blocked by tetrabutylammonium ion.

Relation to inhibition by excess of substrate. Krupka (1963) has shown that inhibition of acetylcholine hydrolysis by excess of substrate is due to the binding of a molecule of acetylcholine to the acetylated enzyme, with consequent blockage of deacetylation. Since this phenomenon is seen in AChE but not in butyrylcholinesterase (Fig. 3a), presumably a binding site exists on AChE which either is lacking in butyrylcholinesterase, or is differently located with reference to the locus of deacetylation.

It seemed possible that it was to this special site in AChE that MCP was bound. The possibility is rendered less likely by the observation (Fig. 3) that acetylcholine provides maximal protection against MCP at a concentration almost one-tenth of that at which inhibition by excess of substrate occurs. More important, the activity of butyrylcholinesterase in hydrolysis of acetylcholine can be inhibited by MCP (Fig. 1), although somewhat less readily; and acetylcholine protects the enzyme against such inhibition (Fig. 3). It therefore appears that the 'extra' binding site of AChE is not involved in binding of MCP.

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REFERENCES

- Adams, D. H. & Whittaker, V. P. (1950). *Biochim. biophys. Acta*, **4**, 543.
Aldridge, W. N. (1950). *Biochem. J.* **46**, 451.
Belleau, B. & Tani, H. (1966). *Molec. Pharmacol.* **2**, 411.
Bracha, P. & O'Brien, R. D. (1968a). *Biochemistry*, **7**, 1545.
Bracha, P. & O'Brien, R. D. (1968b). *Biochemistry*, **7**, 1555.
Brestkin, A. P., Godovikov, N. N., Godyna, E. I., Kabachnik, M. I., Mikhelson, M. Y., Rozengart, E. V. & Yakovlev, V. A. (1964). *Dokl. Akad. Nauk S.S.S.R.* **158**, 880.
Chapman, N. B. & Triggle, D. J. (1963). *J. chem. Soc.* p. 1385.
Gutfreund, H. & Sturtevant, J. M. (1956). *Proc. nat. Acad. Sci., Wash.*, **43**, 719.
Kitz, R. & Wilson, I. B. (1963). *J. biol. Chem.* **238**, 745.
Krupka, R. M. (1963). *Biochemistry*, **2**, 76.
Main, A. R. (1964). *Science*, **144**, 992.
Main, A. R. & Dauterman, W. C. (1963). *Nature, Lond.*, **198**, 551.
Metzger, H. P. & Wilson, I. B. (1963). *J. biol. Chem.* **238**, 3432.
Metzger, H. P. & Wilson, I. B. (1967). *Biochem. biophys. Res. Commun.* **28**, 263.
Purdie, J. E. & McIvor, R. A. (1966). *Biochim. biophys. Acta*, **128**, 590.
Wilson, I. B. (1960). In *The Enzymes*, 2nd ed., vol. 4, p. 50. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
Wilson, I. B. & Bergmann, F. (1950). *J. biol. Chem.* **185**, 479.